Identification and Characterization of a Novel Yeast Gene: the YGP1 Gene Product Is a Highly Glycosylated Secreted Protein That Is Synthesized in Response to Nutrient Limitation

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Nutrient starvation in the yeast Saccharomyces cerevisiae leads to a number of physiological changes that accompany entry into stationary phase. The expression of genes whose products play a role in stress adaptation is regulated in a manner that allows the cell to sense and respond to changing environmental conditions. We have identified a novel yeast gene, YGP1, that displays homology to the sporulation-specific SPS100 gene. The expression of YGP1 is regulated by nutrient availability. The gene is expressed at a basal level during "respiro-fermentative" (logarithmic) growth. When the glucose concentration in the medium falls below 1%, the YGP1 gene is derepressed and the gene product, gp37, is synthesized at levels up to 50-fold above the basal level. The glucose-sensing mechanism is independent of the SNF1 pathway and does not operate when cells are directly shifted to a low glucose concentration. The expression of YGP1 also responds to the depletion of nitrogen and phosphate, indicating a general response to nutrient deprivation. These results suggest that the YGP1 gene product may be involved in cellular adaptations prior to stationary phase and may be a useful marker protein for monitoring early events associated with the stress response.

In the yeast Saccharomyces cerevisiae, a major regulation of cell division occurs in the G1 phase (41). At this point in the cycle, the cell must sense and coordinate both intracellular and extracellular signals before committing to a subsequent round of growth. Upon completion of the cell cycle, cells in a heterothallic haploid culture have two options; they can begin a new round of division, or they can enter stationary phase. When nutrients are limiting, cells exit the division cycle and enter stationary phase. The exact timing of entry into stationary phase and the resulting physiological responses are not well understood.

Yeast cells undergo various physiological changes in response to nutrient depletion (32, 40, 53). Two changes that have been well documented are strengthening of the cell wall and accumulation of trehalose (8, 9, 47, 50). In general, different phases of the growth cycle are marked by major alterations in gene expression. As cells enter stationary phase, the transcription of most genes and the level of mRNA decrease (53). Similarly, there is a substantial drop in overall protein synthesis (4). In contrast, some genes are expressed at higher levels during or after the diauxic shift following "respiro-fermentative" (logarithmic) growth, and in certain cases, the gene products appear to be required for survival under stress conditions (16, 45, 53). The expression of genes that are involved in various stress responses appears to be controlled through Ras-regulated cyclic AMP-dependent protein kinase and Snf1p protein kinase signal transduction pathways (53).

Acid trehalase is one of the enzymes that is derepressed in stationary-phase (i.e., after glucose exhaustion) cells (18). Previously, a protein preparation containing 7,000-fold-purified acid trehalase activity was used to generate antisera (36). The biosynthesis of a highly glycosylated yeast protein that was thought to be the vacuolar trehalase was analyzed in sec mutants (37). To continue analysis of the synthesis and regulation of acid trehalase, we decided to clone the structural gene, relying on data obtained from the purification of the enzyme. In this report, we suggest that the purified protein preparation having high acid trehalase activity was copurified with an additional protein. We provide evidence that this highly glycosylated additional protein does not exhibit acid trehalase activity but is actually a secretory protein, which we call gp37. This protein is the product of a novel yeast gene, YGP1. We have cloned and sequenced the YGP1 gene and examined the regulation of its expression. We have found that YGP1-specific mRNA and the corresponding protein product are present at low levels during respiro-fermentative (logarithmic) growth. The levels of both increase substantially in response to the limitation of several important nutrients. These results suggest that YGP1 may be regulated through a complex mechanism that senses and responds to multiple environmental signals and that the gene product may play a role in cellular adaptations prior to entry into stationary phase.

MATERIALS AND METHODS

Strains and media. The Escherichia coli strains used in this study were MC1061 F- hisD5 RusM* araD139 Δ(araABOIC-leu)769 ΔlacX74 galU galK rpsL (5) and DH5α F- Δ80lacZAM15 Δ(lacZAM15ΔZ)U169 deoR recA1 endA1 hisD17 supE44 Δ- thr-1 gyrA96 relA1. The yeast strains used were SEY2108 Matα ura3-52 leu2-3,112 suc2Δ apr1::LEU2 (2), SEY6211 Matα ura3-52 leu2-3,112 his2Δ200 trpl-Δ901 ade2-101 ycl2Δ200 and SEY6210 Matα ura3-52 leu2-3,112 his3Δ200 trpl-Δ901 lys2-801 suc2Δ9 (42). Standard methods were used to construct yeast strains DKY9001 Matα ura3-52 leu2-3,112 his3Δ200 trpl-Δ901 lys2-801 suc2Δ9 Δsnf1::LEU2, MDY1 Matα ura3-52 leu2-3,112 his3Δ200 trpl-Δ901 lys2-801 suc2Δ9 Δgpl1::URA3, and MDY2 Matα ura3-52 leu2-3,112 his3Δ200 trpl-Δ901 ade2-101 suc2Δ9 Δgpl1::URA3. Plasmid pJH80 (Δsnf1::LEU2), used to disrupt the SNF1 gene (21), was
generously supplied by E. J. A. Hubbard and M. Carlson (Columbia University).

Standard yeast (48, 54) and E. coli (35) media were used and supplemented as needed. The nomenclature of Lewis et al. (31) was used to describe the growth phases of the yeast cultures. Specifically, the term respiro-fermentative refers to the initial logarithmic phase of growth, primarily characterized by the fermentation of glucose. The word logarithmic is included in parentheses throughout the text as a reminder. For specific growth experiments with limiting nutrients, the following media were used. For limiting carbon and energy sources, cells were grown in synthetic minimal medium. This consisted of 0.7% yeast nitrogen base (YNB), amino acids as necessary, vitamins, and 2% glucose. To test for growth on nonfermentable carbon sources, the glucose concentration was reduced to 0.05% and the medium was supplemented with 3% glycerol-lactate, 2% pyruvate, or 2% ethanol. To analyze expression with limiting phosphate, cells were grown in 50 mM 2-(N-morpholino)ethanesulfonic acid (pH 5.5)−1% asparagine−10 mM MgSO₄−27 mM KCl−4.5% glucose−vitamins−trace elements (including 0.002 mM FeCl₃)−0.002 to 2 mM potassium phosphate. For limiting sulfate, cells were grown in 7.3 mM potassium phosphate−1.7 mM NaCl−4.1 mM MgCl₂−vitamins−trace elements−2% asparagine−4.5% glucose−0.001 to 10 mM MgSO₄. For experiments with limiting nitrogen, the medium consisted of 7.3 mM potassium phosphate, 1.7 mM NaCl, 4.1 mM MgCl₂, 10 mM MgSO₄, vitamins, trace elements, 5% glucose, and 0.002 to 0.2% asparagine.

Reagents. YNB, Bacto Tryptone, Bacto Peptone, Bacto Yeast Extract, and Bacto Agar were from Difco Laboratories (Detroit, Mich.). DNA restriction and modifying enzymes were from New England Biolabs, Inc. (Beverly, Mass.), and Boehringer Mannheim Biochemicals (Indianapolis, Ind.). Zymolyase 100 T was purchased from ICN Biomedicals (Irvine, Calif.), oxalate was obtained from Enzymogenetics (Corvallis, Oreg.), endoproteinase Lys-C was obtained from Boehringer GmbH (Mannheim, Germany), and endoglycosidase H was obtained from Boehringer Mannheim Biochemicals. Hybrid N⁺ membranes for Southern and Northern (RNA) blots, [α-32P]-dCTP (3,000 Ci/mmol), and [35S]-dATP (1,000 Ci/mmol) were from Amersham Buchler (Braunschweig, Germany). Polynylidene difluoride membranes for Western blots (immo-blot) (Immobilon-P) were from Millipore Corp. (Bedford, Mass.). Goat anti-rabbit antibodies were purchased from Bio-Rad Laboratories (Hercules, Calif.), and the ECL chemiluminescence detection kit was obtained from Amersham (Arlington Heights, Ill.). Sequenase 2.0 and random priming kits were from United States Biochemical Corp. (Cleveland, Ohio). All other chemicals were from Sigma Chemical Co. (St. Louis, Mo.).

Purification of acid trehalase-gp37, proteinase digestion, and peptide sequencing. Purification of acid trehalase-gp37 was carried out as previously described (36). A portion (100 μg) of the purified preparation was deglycosylated with endoglycosidase H as described below. Half of the deglycosylated protein was digested with endoproteinase Lys-C, which cleaves on the carboxyl side of lysine residues, and used to determine the sequences of internal peptide fragments by the Edman degradation method. The remaining portion was also subjected to Edman degradation to determine the amino-terminal sequence of the mature protein. The proteinase digestion and protein sequencing reactions were performed by H. E. Meyer (Ruhr Universität, Bochum, Germany).

Cloning, sequencing, and DNA analysis of YGPI. Peptide sequences from the amino terminus and an internal peptide of the acid trehalase-gp37 protein were used as the basis for synthesizing degenerate oligonucleotides (7). The oligonucleotides were used in a PCR with yeast genomic DNA isolated from strain SEY6210. A specific 0.5-kbp DNA fragment was amplified and recovered. This fragment was labeled with [32P]-dCTP by the random priming method (14) and used to screen a YCP50-based genomic DNA library (43) by colony hybridization (44). Four plasmids that hybridized with the probe were detected. These plasmids contained a common 1.9-kbp HindIII restriction enzyme fragment. This fragment was subcloned into vector pTZ18R (Pharmacia, Freiburg, Germany) to construct plasmid pTZYGPI. The nucleotide sequence of both strands of the 1.9-kbp fragment containing the YGPI gene was determined by the dideoxy chain termination method (46).

Genomic and plasmid DNAs from S. cerevisiae and plasmid DNA from E. coli were prepared as described previously (3, 48). Standard procedures were followed for subcloning DNA fragments and for identifying recombinant clones (44). Southern blot analysis (49) was done with radiolabeled DNA hybridization probes prepared by the random priming method as described above. The DNA was separated on an 0.8% agarose gel, incubated in 0.25 M HCl, and blotted onto a Hybond N⁺ membrane in 0.4 M NaOH.

Disruption of YGPI. Plasmid pTZYGPI contains a unique Hpa1 site within the YGPI structural gene. A 1.1-kbp HindIII fragment containing the URA3 gene was isolated from plasmid YEp24, and the overhanging 5' ends were filled in by treatment with the Klenow fragment of DNA polymerase I. The blunt-ended URA3 fragment was cloned into the Hpa1 site of pTZYGPI to generate plasmid pTZYGPI1. The HindIII fragment of pTZYGPI1 containing the YGPI gene disrupted with URA3 was isolated and used to transform yeast strains SEY6210 and SEY6211 with approximately 10 μg of DNA (22). Ura⁺ colonies were isolated and examined by Southern blotting to confirm the site of integration. Yeast strains MDY1 and MDY2 contained the URA3 gene integrated at the chromosomal YGPI locus.

RNA isolation and Northern blot analysis. Total RNA was isolated from respiro-fermentative (logarithmic) and respira-
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FIG. 2. Nucleotide sequence of the nontranscribed strand of the YGPI gene. The nucleotide sequence was obtained for both strands of YGPI by the method of Sanger et al. (46) with two subclones in vector pTZ18R. Two putative transcription initiation sequences (TATA boxes) in the 5′ untranslated region are doubly underlined, and the putative 3′ polyadenylation signal is underlined and italicized. Nucleotide 1 corresponds to the first nucleotide of the initiation codon. Translation of the predicted open reading frame of the YGPI gene is shown below the nucleic acid sequence. Amino acid 1 corresponds to the first amino acid of precursor gp37. Potential sites for asparagine-linked glycosylation are indicated in boldface type. The amino acid sequences derived from protein sequencing are underlined, and sequences used for the design of oligonucleotide primers are underlined and italicized.

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0.27 M sodium acetate (pH 5.2)–0.5% sodium dodecyl sulfate (SDS)–0.5% β-mercaptoethanol. When needed, protein was deglycosylated by denaturation at 95°C for 5 min in 0.27 M sodium acetate (pH 5.2)–0.5% SDS–0.5% β-mercaptoethanol and then treatment with endoglycosidase H (20 mU) for 48 h at 37°C. An aliquot of the crude extract or deglycosylated protein (35 to 50 μg) was loaded onto a 10% polyacrylamide–SDS gel. Following electrophoresis, the proteins were transferred to a polyvinylidene difluoride membrane by electroblotting. The filter was blocked and incubated for 14 h with a 1:20,000 dilution of antibody to gp37. Antibody binding was visualized by subsequent incubation with horseradish peroxidase-conjugated goat anti-rabbit antibody and ECL detection reagents and then chemiluminescent exposure of X-ray film.

**Hybrid protein construction.** Sall restriction enzyme sites were placed after amino acid residue 39 or 354 of precursor gp37 by PCR. An EcoRI restriction enzyme site was placed in the 5’ noncoding region at nucleotide positions –353 to –348 (see Fig. 2) by PCR. The SUC2 fusion vector pSEYC308 was described previously (26). The EcoRI-Sall-amplified DNA fragments were cloned into the unique EcoRI and Sall sites of pSEYC308 to generate hybrid proteins GPI-39 and GPI-354. The construction of the PH08-SUC2 gene fusion containing the amino-terminal 191 amino acids of alkaline phosphatase fused to invertase, API-191, was described previously (27). The PEP4-SUC2 gene fusions, P41-23 and P41-137, containing portions of protease A fused to invertase, were described previously (26).

**Assays.** Invertase and glucose detection assays were done as described previously (17). At each time point to be assayed, approximately 2 volumes of cells at an optical density at 600 nm of 1.0 was centrifuged and the supernatant fraction was removed for determination of the remaining glucose concentration. The cells were washed in 10 mM sodium azide, centrifuged, and resuspended in 0.5 ml of 0.1 M sodium acetate (pH 5.0). An aliquot of the resuspended cells was removed to determine the optical density of the resuspended culture, and the remaining cells were lysed by the addition of Triton X-100 (0.5% final concentration) and freezing in dry

FIG. 2—Continued.

```plaintext
613  CCT GTT GCT ATC GTT GAT GAC CAA GAC GAA GAA GTT AAA TGG TTC TTC GAT GCT
205  Pro Val Ala Ile Val Asp Asp Gln Val Val Lys Trp Phe Phe Asp Ala
664  TCT AAG CCA ACT TTA ATC TCT TCT GAC TGG ATT ATC AGA AAG GAA TAC AGT
222  Ser Lys Pro Trp Leu Ile Ser Asp Ser Asp Ser Ile Arg Lys Tyr Ser Ser
715  AAC TTC ACT ACT CCT TAT GGT CTA TTA GAA AAC GGT GTC CCA ATT GTT CCA
239  Asp Phe Thr Thr Pro Tyr Gly Leu Glu Asn Gly Val Pro Val Pro
766  ATT GTC TAT GAC GGT GTG TAC TCT CAG TGG ATT GAC TCC TGG AGT TCT
256  Ile Val Tyr Asp Gly Gly Tyr Ser Ser Ser Leu Asp Ser Leu Ser Ser
817  GCC GCT GAA GGT TTT GGT TCT TCT GCT GCT GCC AGT TCT CTA ACC ACA ACC TCA
273  Ala Val Gln Gln Leu Leu Val Val Ser Gly Ser Thr Ser Ser Thr Thr Ser Ser
868  TCT ACT ATT GAA AGC ACT GAA ACT CCA GTC GTA TAT GCT GAA CAC ACT Ser Thr Ile Ser Thr Glu Ser Thr Ser Thr Thr Ser
290  Pro Leu Asp Phe Ile Ser Asp Asp Asp Leu Gln Val Val Phe Pro Val Tyr Ala Gln Ala Asn Thr
919  CCA TTA AAC TTG ATT GAC AAC AAG GAT GTC CCA AAG AAG CAC ACT Ser Thr Ile Ser Thr Glu Ser Thr Ser Thr Ser Thr Ser
307  Pro Leu Asn Phe Ile Ser Asp Asp Asp Leu Gln Val Val Phe Pro Val Tyr Ala Gln Ala Asn Thr
970  GGT TAC CTA GCC ATT AAG GCC CAA ATC TTT GGT TAC TCT CTT ATT GCC GTT
324  Gly Tyr Leu Pro Ser Pro Lys Ala Glu Ile Leu Leu Ser Ile Ala Ala Val
1021 AAT GGT GTC ACC TCC AAG CTC CTT CGT GCT TGT GAG ACC ATT TTC ACT GAT
341  Asn Gly Val Thr Ser Lys Ser Ala Leu Ser Glu Ile Phe Pro ***
1072 AGATATAAAA TCTAGGCAAG CATAGAATT CTTTTTATAC GACATCATAC ACCATATT
1132 TTACAAAATT TCCAGCGCAAC AGCTTATATT TTCTTCCGTA ATAGGAAACCA TACTCCCATAT
1192 TAAAGGATATA TGAGTTTTTT ATACACCTTT TTAATGTAT TTAAGACATA CCCCAAATAAA
1252 TTAAGTATTAT AATGATGCAA AGAATCATAT AAATCTTTGG CCATACATAT TTTTTTATTA
1312 TACCTATCTA CATGGGACATT AGGTTGAAAT CTCTCCCGGG TGACGCTCCCA CTGAAGGAGG
1372 AAAAAAGAA AAGTTTTTAA AGATTGGTCA CCTTAAGTCT CGAAGGCTCA GCATTCTACA
1432 CCTGTAACTG TCAAGGCCCC ACAGTGATAC ATGATACAT AAGAAAACCAA TGCTCGTATA
1492 ATGATGATA TTTTACTACA GATAAGGGAT CTACACCCCC TCTTTTTGTT CTCAAAGGAA
1552 TCTCTGATA TATTTAGGTT TTATAGGCT CCAAGTTTTTA AACTTAAAGCC GCAAATAAGT
1612 AGTGTGATGA AACGCTTAT TCTGAGACAT CAGTTAAAA GATATTTTAA TTCTACACTC
1672 AATGCCATTTA GTGAGTGGGCA TTTATGCTA ATTCCACTACA ATTTTACAAA ATTTTTAGAAC
1732 CCAAGACGG CCTAATGAGTT GTTATATAC GTTATAGGCT TCAAGGCTCA CAGTACGAAA
1792 AGCTT
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RESULTS

Cloning and sequencing of YGP1. Previous studies of the vacuolar enzyme trehalase led to the purification and initial characterization of a highly glycosylated protein that was shown to passage through the secretory pathway (36, 37). To further characterize this enzyme, we initiated experiments designed to clone the gene, designated ATPI, encoding this vacuolar hydrolase. As a first step, a protein preparation having high acid trehalase activity was purified from the yeast S. cerevisiae as described previously (36). Highly glycosylated proteins such as acid trehalase and invertase are difficult to separate under the conditions used in the purification, necessitating the use of a sac2 mutant strain. The protein purification procedure resulted in a 7,174-fold increase in the specific activity of acid trehalase (36). It appears, however, that additional proteins still may have been retained during the purification (see below).

Four peptide fragments derived from endoproteinase Lys-C digestion of the purified protein preparation were sequenced by Edman degradation. On the basis of the sequence information, degenerate oligonucleotides corresponding to the amino terminus and an internal peptide fragment were prepared (Fig. 1). Amplification of yeast genomic DNA by PCR produced a 500-bp fragment that contained a single open reading frame. The open reading frame encoded both of the peptide sequences upon which the degenerate oligonucleotides were based. To recover the full-length gene, the 500-bp fragment was used to probe a genomic DNA library as described in Materials and Methods. Four plasmids that had an overlapping 1.9-kbp HindIII restriction enzyme fragment were obtained by colony hybridization. The sequence of the 1.9-kbp fragment is shown in Fig. 2, along with additional sequence information from the 5′ noncoding region.

The entire 1.9-kbp fragment has a single open reading frame of 1,062 bp. The 5′ noncoding region contains two possible TATA boxes, and there is a putative polyadenylation signal 340 bp downstream of the stop codon in the 3′ untranslated region. The open reading frame encodes a 354-amino-acid protein with a predicted molecular mass based on the deduced amino acid sequence of approximately 37,381 Da. The first 20 amino-terminal residues have a sequence which fits with the normal hydrophobic consensus sequence for signal peptides. In addition, there are two sequences in this region that contain possible signal sequence cleavage sites, on the basis of the rules of von Heijne (52). The site with the highest probability of cleavage is between amino acid residues 19 and 20 (S value of 8.66). The coding region contains 14 potential N-glycosylation sites. Eleven of these are the more commonly used Asn-X-Thr sequence (38), suggesting that the protein might undergo extensive carbohydrate modification. For reasons described below, the cloned gene did not appear to encode the vacuolar acid trehalase. The gene was named YGP1, for yeast glycoprotein. On the basis of the deduced molecular mass and glycosylation (see below), the gene product was named gp37, for 37-kDa glycoprotein.

The YGP1 gene does not encode acid trehalase. The peptides that were the basis for the degenerate oligonucleotides used in the cloning of YGP1 were derived from a protein preparation having high acid trehalase activity (36). We refer to this protein preparation as acid trehalase-gp37. For several reasons, we believe that YGP1 is not the structural gene for acid trehalase. Acid trehalase is a vacular protein (25, 33, 36). None of the well-characterized vacular proteins show the extensive glycosyl modification that are typical of proteins such as invertase and acid phosphatase (1, 28, 29). The protein characterized by

Ice. Invertase assays were performed with 0.0025 to 0.2 optical density unit of cells. One optical density unit of cells corresponds to approximately 10⁷ yeast cells or 200 µg of protein.

To quantitate the secretion of hybrid proteins, intact and detergent-permeabilized cells synthesizing the GPl-39, GPl-354, P41-23, or P41-137 hybrid protein were assayed for invertase activity (26). Yeast cells were harvested in mid-respiro-fermentative (mid-logarithmic) or respiratory phase and collected by centrifugation. The cells were washed in 10 mM sodium azide, resuspended in 0.1 M sodium acetate (pH 5.1), and divided into two aliquots. A sample was measured to determine the optical density. One aliquot was kept on ice (intact cells). Triton X-100 (0.5% final concentration) was added to the remaining aliquot, which was then frozen-thawed in dry ice (lysed cells). Invertase assays were carried out, and the percentage of secreted invertase was determined by dividing the activity detected in intact cells by the total activity detected in lyes cells.

The percentage of ethanol in the growth media was determined by chromatography on a Hewlett-Packard 5710A gas chromatograph with a Supelco TintSpec glass column with 80/120 Carbopack B AW packing and 5% Carbawax 20M coating.

Nucleotide sequence accession number. The sequence of the YGP1 gene has been assigned EMBL number X73030 (S. cerevisiae YGP1 gene).

![Western blot analysis of wild-type and ygp1 mutant strains.](image)
Mittenbuhler and Holzer (36, 37) is highly glycosylated and migrates with an apparent molecular mass of 220 kDa. This type of extensive glycosylation would be unexpected for vacuolar acid trehalase. Similarly, native gp37 migrated as a high-molecular-weight smear on SDS-polyacrylamide gels (Fig. 3, lane 1). This mobility is characteristic of highly glycosylated proteins and likely reflects a heterogeneous mixture of gp37 molecules with variable-length outer mannoside carbohydrate chains. Deglycosylation of gp37 produced a discrete species which migrated at approximately 41 kDa (Fig. 3, lane 2). This apparent molecular mass is in close agreement with the size of the YGPJ gene product predicted on the basis of the deduced amino acid sequence.

To further test the identity of gp37 and acid trehalase, we constructed a yeast strain that had a disruption of the chromosomal YGPJ gene. A strain disrupted at the YGPJ locus, MDY1 (ΔygpJ::URA3), was constructed by insertion of the URA3 gene at the HpaI site of YGPJ as described in Materials and Methods. The correct integration was confirmed by Southern blotting (data not shown). The disrupted mutant was fully active for acid trehalase (8a). The YGPJ locus in strain MDY1 was disrupted with a gene insertion, and none of the gene sequence was deleted. This means that it is possible that the activity corresponding to the gene product has not been eliminated. We consider this possibility unlikely, however, since gp37 was not detected in protein extracts from this strain (Fig. 3, lanes 3 and 4). Similarly, antibodies prepared against gp37 do not precipitate acid trehalase activity from the yeast cytosol (8a).

Finally, we present evidence (see below) that gp37 is a secreted protein, a fact which is not consistent with it being localized in the vacuole. Perhaps as the result of similar physical properties, such as a high degree of glycosylation or the nature of acid trehalase as a sugar-binding protein, acid trehalase and gp37 may have been copurified in the preparation described by Mittenbuhler and Holzer (36). The endoprotease Lys-C-generated peptides obtained from the acid trehalase-gp37 purification and upon which the degenerate oligonucleotides used to clone YGPJ were based are probably derived from gp37 and not from acid trehalase. On the basis of these data, we believe that YGPJ is not the structural gene for acid trehalase and that gp37 is not the vacuolar acid trehalase.

YGPJ is homologous to a sporulation-specific protein. The amino acid sequence deduced from the YGPJ gene was compared with those of proteins in the Swiss-Prot and PIR protein data bases by use of the FASTA algorithm and the Wordsearch program of the University of Wisconsin Genetics Computer Group Package (10). This analysis revealed significant homology between gp37 and a sporulation-specific protein that is the product of the SPS100 gene (30). These two proteins show 50% identity and 67% overall similarity over their entire lengths (Fig. 4). The similarity is particularly striking over the C-terminal halves of the two proteins. The SPS100 gene was identified by screening with sporulation-specific RNA probes to identify genes that are expressed near the completion of sporulation. SPS100 RNA appears 35 h after the transfer of diploid cells to sporulation medium (48). A mutation in SPS100 resulted in one detectable phenotype; spores from the mutant strain were less resistant to diethyl
either, suggesting a role for the gene product in spore wall formation.

**Expression of YGPI is subject to glucose repression.** Because of the homology of YGPI with SPS100, we decided to examine expression of the YGPI gene during different phases of growth. We examined expression of the YGPI gene during respiro-fermentative (logarithmic) and respiratory phases by determining the levels of YGPI-specific mRNA present at various time points of growth (Fig. 5). Cells were grown in YP medium (1% Bacto Yeast Extract, 2% Bacto Peptone) containing 2% glucose. At various times, aliquots of cultures were removed and the presence of YGPI-specific mRNA was analyzed by Northern blotting as described in Materials and Methods. The YGPI gene is expressed at low levels during respiro-fermentative (logarithmic) growth (Fig. 5, lanes 1 and 2). Exit from this phase of growth and entry into the respiratory phase (marked by the depletion of glucose and growth on ethanol) in a haploid strain is accompanied by an increased level of expression of YGPI-specific RNA, as revealed by Northern blotting (Fig. 5, lanes 3 to 5). The bulk 18S and 25S rRNA species served as controls and showed that essentially equivalent amounts of RNA were analyzed at each time point. Since YGPI-specific RNA is not expressed at high levels in cells during respiro-fermentative (logarithmic) growth, we decided to examine the synthesis of the gp37 protein during different growth phases. To examine the synthesis of gp37, protein extracts were prepared from yeast cells at different stages of growth and analyzed by Western blotting. We found that the protein was present at a low level in extracts prepared from logarithmically growing cells (Fig. 6, lanes 2 to 4). In contrast, when respiratory-phase cells were examined, there was a substantial increase in the level of gp37 (Fig. 6, compare lanes 4 and 5). This result correlated with the Northern blot analysis of YGPI-specific RNA and suggested that the regulation of expression occurred primarily at the translational level rather than at the translational level.

The Western blot analysis indicated that the synthesis of gp37 is subject to repression by high glucose concentrations. To carefully quantify the reduction in glucose concentration that is necessary to allow the expression of YGPI, we examined the regulation of expression through the use of hybrid proteins. Portions of YGPI encoding either the first 39 amino acids (GPI-39) or the full-length protein (GPI-354) were fused to a fragment of the yeast SU2 gene, encoding invertase, as described in Materials and Methods. The truncated SU2 gene that is present in these constructs lacks the promoter region
and amino-terminal signal sequence. In the gene fusions, the expression of invertase activity is therefore controlled by the YGP1 regulatory elements. Plasmids encoding the hybrid proteins were transformed into a strain bearing a deletion of the chromosomal SUC2 locus.

The results obtained with the hybrid proteins were in agreement with the Northern blot analysis of YGP1-specific mRNA (Fig. 5) and our observations of the authentic gp37 protein (Fig. 6). During logarithmic growth, a basal level of invertase activity corresponding to either the GPI-39 or the GPI-354 hybrid protein was seen (Fig. 7A). When the level of glucose in the medium dropped to 1%, increased invertase activity was detected (Fig. 7B). This result corresponded to the Northern blot analysis, which indicated that YGP1-specific mRNA was detectable only when the glucose level dropped from 1.6 to 0.96% (Fig. 5). The invertase activity from the gp37-invertase hybrid proteins continued to increase during the diauxic lag phase and remained steady during part of the respiratory phase. The invertase activity peaked approximately 60 to 80 h after growth had reached a plateau (Fig. 7D). The maximal level of activity seen was approximately 50-fold above the basal level.

We decided to determine whether the increased activity was the result of de novo protein synthesis. Cultures of yeast cells harboring the plasmid encoding the GPI-354 hybrid protein were grown in synthetic medium with 2% glucose. When the glucose level dropped below 1%, cycloheximide was added to one of the cultures. Invertase activity did not increase after the addition of cycloheximide (Fig. 7D). Instead, there was a slow decrease in activity that may reflect the normal turnover of gp37.

When cells exhaust a limiting nutrient, they may enter stationary phase. Entry into stationary phase results in changes that provide increased resistance to stress. These physiological adaptations, however, do not necessarily depend on the growth phase of the cells but rather depend on the growth rate (13). To determine whether the expression of YGP1 was triggered by some aspect of the growth rate or by glucose depletion, we grew yeast cells under conditions in which glucose was present in excess and growth was limited by the availability of an auxotrophic amino acid.

A yeast strain auxotrophic for histidine was grown in synthetic medium containing either limiting glucose and the necessary amino acids or a nonlimiting concentration of glucose and a limiting concentration of histidine. When the auxotrophic amino acid became limiting, the cells ceased growing (Fig. 7E and F). The glucose level in this culture did not drop below 1%. Under these conditions, YGP1-dependent expression of invertase did not increase. This result suggests that the increase in invertase activity regulated by the YGP1 promoter does not correspond to slow growth or a cessation of growth per se but rather corresponds to glucose depletion. In addition, the increase in activity is not due to nonspecific starvation effects. Alkaline phosphatase, the product of the PHO8 gene (24, 51), is regulated by phosphate through the interaction of several PHO genes (reviewed in reference 39). An alkaline phosphatase-invertase hybrid protein does not show elevated expression upon glucose depletion (Fig. 7C).

Expression of YGP1 is not regulated by the general glucose control of the SNF1 pathway. Many genes that are subject to catabolite repression can be derepressed upon shifting of the

FIG. 8. The YGP1 gene is regulated by the phosphate concentration. Yeast strain SEY2108 transformed with a plasmid encoding the GPI-354 hybrid protein was grown as described in Materials and Methods in minimal medium with initial potassium phosphate concentrations ([PO₄]₀) of 0.002 to 2 mM. Cultures were inoculated to an initial optical density at 600 nm (O.D₆₀₀) of approximately 0.01, and samples were removed at the indicated time points. At each time point, an aliquot of the culture was removed, and the invertase activity corresponding to the GPI-354 hybrid protein and the glucose concentration in the medium were determined as described in Materials and Methods. Invertase activity is expressed as nanomoles of glucose released per minute per optical density unit of cells.
growing culture to a medium containing nonrepressing levels of glucose. We tested the effect of this type of acute change in glucose levels on the expression of YGP1. Yeast cells harboring a plasmid encoding GPI-354 were grown in YNB medium with 2% glucose. During respiro-fermentative (logarithmic)-phase growth, portions of the culture were removed and the cells were collected by centrifugation and resuspended in fresh YNB medium with 0.05 or 0.1% glucose. The cells were incubated for an additional 136 h, and samples were removed at 12 h intervals to assay for invertase activity. We found that the expression of YGP1 was not increased when cells were shifted directly to a medium containing a low level of glucose (data not shown).

The absence of the elevated expression of YGP1 when cells were shifted to a medium containing a low level of glucose suggests that the gene may be regulated by a unique type of glucose repression. We tested whether YGP1 was regulated by the general glucose control pathway, which utilizes SNF1 (23). A yeast strain disrupted at the chromosomal SNF1 locus was transformed with a plasmid encoding the GPI-354 hybrid protein. Cells were grown beyond the point of glucose depletion and assayed for invertase activity. In both wild-type and snf1 mutant cells, the level of invertase activity from the GPI-354 hybrid protein increased when glucose was depleted from the medium (data not shown). In the snf1 strain, the activity reached a level 50 to 60% of that seen in the SNF1 (wild-type) strain. This level of invertase activity corresponds to an approximately 17-fold increase above the basal level of invertase activity seen during respiro-fermentative (logarithmic)-phase growth. In a control experiment, snf1 mutant cells were unable to derepress the synthesis of invertase from a plasmid encoding the wild-type SUC2 gene under the control of its endogenous promoter (data not shown). The derepression of YGP1 is apparently not dependent on a functional SNF1 gene product.

Expression of YGP1 is elevated in response to multiple signals. To determine whether the expression of YGP1 is regulated only by the glucose concentration or by a more general nutrient limitation, we examined the expression of gp37-invertase hybrid proteins under conditions of limiting

FIG. 9. Limiting sulfate or slow growth does not allow the expression of YGP1. Yeast strain SEY2108 transformed with a plasmid encoding the GPI-354 hybrid protein was grown as described in Materials and Methods in minimal medium with the initial potassium phosphate ([PO$_4^-$]) or magnesium sulfate ([SO$_4^{2-}$]) concentrations indicated. Cultures were inoculated to an initial optical density at 600 nm (O.D._600) of approximately 0.005 to 0.01, and samples were removed at the indicated time points. Aliquots of the culture were assayed for invertase activity and glucose concentration as described in the legend to Fig. 8. The glucose level in the medium varied from 3.8 to 4.5% over the indicated time course for all of the cultures shown. Invertase activity is expressed as nanomoles of glucose released per minute per optical density unit of cells.
The YGPI gene is regulated by the nitrogen concentration. Yeast strain SEY2108 transformed with a plasmid encoding the GPI-354 hybrid protein was grown as described in Materials and Methods in minimal medium with initial asparagine concentrations ([Asn]) of 0.002 to 0.2%. Aliquots of the culture were assayed for invertase activity and glucose concentration as described in the legend to Fig. 8.

FIG. 10. The YGPI gene is regulated by the nitrogen concentration. Yeast cells harboring the plasmid encoding GPI-354 were grown in modified Wickerham's minimal medium (54) containing 4 to 5% glucose and various phosphate, sulfate, and nitrogen concentrations as described in Materials and Methods. Samples were removed at various times during growth corresponding to respiro-fermentative (logarithmic), diauxic lag, and respiratory phases of growth. Cells at each time point were assayed for invertase activity to assess the expression of YGPI, and the medium was assayed to determine the glucose concentration.

The expression of YGPI was not dependent solely on the level of glucose. Expression increased in a time-dependent manner at initial phosphate concentrations of 2 to 20 μM, even though the glucose concentration did not drop below approximately 4% (Fig. 8). The maximal level of invertase activity was higher than that seen when cell growth was limited only by glucose. The highest level of YGPI expression was seen at 0.2 mM initial phosphate. In this case, the invertase activity began to increase before the glucose concentration fell below 1%. In contrast to the results obtained with the 0.002 to 0.02 mM phosphate-starved cultures, however, with a 0.2 mM initial phosphate concentration there was sufficient phosphate to allow growth to eventually deplete the glucose to below the 1% level (Fig. 8B). This may have caused a synergistic effect resulting in the maximal expression of YGPI to a level approximately 200-fold above the basal level. At an initial phosphate concentration of 2 mM, the level of expression of YGPI increased only after the glucose concentration fell below 1%.

In contrast to the results obtained with limiting phosphate, starvation for sulfate did not cause an increase in YGPI expression over the time course examined (Fig. 9). Even though the sulfate- and phosphate-starved cultures showed similar doubling times and growth yields, there was a significant difference in the levels of invertase activity from the GPI-354 hybrid protein in cells grown under the two conditions. This result suggests that growth phase is not determinant of YGPI expression, in agreement with the results obtained with limiting histidine (Fig. 7E).

Like phosphate depletion, nitrogen depletion allowed the expression of YGPI (Fig. 10). When asparagine was present as the sole nitrogen source at an initial level of 0.002%, there was an approximately tenfold increase in the level of invertase activity above the basal level. This relatively low level of expression was probably due to the overall poor growth of this culture. At 0.02% initial asparagine, the level of expression of YGPI increased approximately 35-fold, even though the glucose concentration remained above 1%. These results suggest that YGPI is responsive to the limitations of multiple nutrients, including phosphate and nitrogen, in addition to carbon and energy sources.

The gp37 protein is degraded in the presence of glucose. The synthesis of certain enzymes is repressed by glucose in a process termed catabolite or glucose repression. Many enzymes which are regulated by glucose are also subject to glucose-induced inactivation, which is sometimes referred to as catabolite inactivation (19). This refers to the inactivation of preexisting enzymes as opposed to the inhibition of new synthesis. This type of nutrient regulation is often the result of proteolysis (20). To examine the possibility of proteolytic inactivation of gp37, cells were grown for 48 h to deplete the glucose and allow expression of the protein. Glucose was added back to the culture to a final concentration of 3%, and the cells were allowed to continue growing. Samples were taken at different time points and used to prepare protein extracts. The extracts were deglycosylated with endoglycosid-
dase H as described in Materials and Methods and analyzed by Western blotting (Fig. 6).

The gp37 protein was degraded in the presence of glucose. Within 8 h after the addition of fresh glucose to the glucose-depleted cultures, there was no detectable gp37. When the glucose had again been depleted from the medium following renewed growth, gp37 was resynthesized. In control cells, which did not receive additional glucose, the level of gp37 remained relatively unaffected. The degradation of gp37 seen in the presence of glucose was probably due to a specific process of nutrient regulation involving proteolysis and was not likely to reflect the normal rate of turnover of the protein in the absence of new synthesis. When the synthesis of gp37 was inhibited by cycloheximide, the protein was degraded at a greatly reduced rate (Fig. 7D); following the addition of cycloheximide, there was a 50% reduction in invertase activity in approximately 48 h. Since certain types of proteolytic inactivation may be blocked in the presence of cycloheximide (15), the decrease in invertase activity seen in the presence of this antibiotic may have been an indication of the normal rate of turnover of gp37.

The mechanism by which glucose regulates the expression of YGP1 appears to be complex, since derepression did not occur upon shifting of cells to a low glucose concentration. To better understand the phenomenon of glucose repression, we examined the expression of YGP1 during growth with various nonfermentable carbon and energy sources. Yeast cells harboring the GPI-354 hybrid protein were grown in YNB medium containing either glucose, glycerol-lactate, pyruvate, or ethanol as described in Materials and Methods. At various time points, aliquots were removed and assayed for invertase activity. In addition, the media were assayed to determine the glucose and/or ethanol levels. Growth with glycerol-lactate or pyruvate did not allow the expression of YGP1 (Fig. 11A).

In contrast to cells grown with glycerol-lactate and pyruvate, cells grown with ethanol showed an approximately 100-fold increase in invertase activity above the basal level (Fig. 11A). The observed increase did not reflect derepression in the usual sense, in that the increase in activity did not occur for approximately 3 days following growth with ethanol. The increase in the level of expression occurred when the ethanol level dropped to 0.8 to 1% (Fig. 11C). It should be noted that

![Graph](http://mcb.asm.org/)
TABLE 1. Secretion of hybrid proteins

<table>
<thead>
<tr>
<th>Hybrid protein</th>
<th>Invertase activity (U) in:</th>
<th>% Secreted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intact cells</td>
<td>Lysed cells</td>
</tr>
<tr>
<td>GPI-354*</td>
<td>12</td>
<td>14</td>
</tr>
<tr>
<td>GPI-354</td>
<td>172</td>
<td>209</td>
</tr>
<tr>
<td>GPI-39</td>
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<td>221</td>
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<tr>
<td>P41-23</td>
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<td>515</td>
</tr>
<tr>
<td>P41-137</td>
<td>21</td>
<td>319</td>
</tr>
</tbody>
</table>

\* The invertase assay was performed on cells in respiro-fermentative (logarithmic)-phase growth. All other assays were carried out on respiratory (post-glucose exhaustion)-phase cells.

when cells were grown with 2% glucose, the ethanol level remained below the 0.8 to 1% range (Fig. 11B).

Localization of gp37. For the reasons described above, we believe that Mittenbühler and Holzer (37) may have partly characterized the biosynthesis of gp37 rather than acid trehalase. Their results, when taken for gp37 instead of acid trehalase, indicate that gp37 is highly glycosylated, as expected from the number of potential glycosylation sites. This conclusion was confirmed by the current study, which demonstrated that gp37 migrated as a heterogeneous highly glycosylated protein (Fig. 3). In fact, gp37 is one of the most highly glycosylated yeast proteins on a weight-to-weight basis. The apparent molecular weight of gp37 (36, 37) (Fig. 3) and the number of potential glycosylation sites suggested that there may be an average of approximately 90 mannose residues per carbohydrate side chain. In addition, Mittenbühler and Holzer (37) showed that passage of the protein through the secretory pathway is dependent on the products of certain SEC genes. Intermediate-molecular-weight forms of the protein accumulate in sec61, sec18, and sec7 mutant cells at the nonpermissive temperature (37).

As discussed earlier, gp37 is predicted to have a signal sequence cleavage site between amino acid residues 19 and 20. The mature protein, however, is proposed to begin at residue 38, on the basis of amino acid sequencing of the purified protein. This result may indicate additional processing subsequent to the removal of the signal sequence or possible degradation during the purification procedure.

We examined the location of the gp37-invertase hybrid proteins in yeast cells that were repressed or derepressed for the expression of YGP1. Cells harboring a plasmid encoding either the GPI-39 or the GPI-354 hybrid protein were grown in synthetic medium containing 2% glucose. In addition, we analyzed two control proteins, P41-23 and P41-137, which contain portions of proteinase A fused to invertase (26). P41-23 has only the signal sequence portion of proteinase A and serves as a control for a secreted protein. P41-137 includes a segment of proteinase A that contains the vacuolar sorting information. This hybrid protein is efficiently targeted to the vacuole and provides a control for a nonsecreted protein.

Samples of cells were removed during respiro-fermentative (logarithmic)-phase or respiratory-phase growth and assayed for secreted versus total invertase activity as described in Materials and Methods. In either case, approximately 80 to 100% of the invertase activity corresponding to the gp37-invertase hybrid proteins was secreted into the periplasm (Table 1). Immunoprecipitation of radiolabeled cells indicated that the hybrid proteins were stable for at least 1 h (data not shown). Since secretion in yeast cells is rapid, the above-described results obtained with GPI-39 and GPI-354 reflect the localization of gp37 and not that of a degraded hybrid protein. This extracellular localization further suggested that YGP1 does not encode the vacuolar enzyme acid trehalase. The extracellular localization also fits with the extensive glycosylation seen for gp37, which is similar to that of certain other secreted proteins (1, 29).

DISCUSSION

We have identified a novel yeast gene, YGP1, that encodes a highly glycosylated secretory protein. The YGP1 gene is subject to glucose repression. The expression of YGP1-specific RNA and the synthesis of the gene product, gp37, occurred at basal levels until the glucose concentration of the medium dropped below 1%. An apparently similar change in expression occurred when cells were grown in ethanol-based medium and the ethanol concentration dropped below approximately 1%. Derepression of YGP1 was also seen when cells were grown under conditions in which phosphate or nitrogen became limiting. The mechanism(s) by which these nutrient signals are transduced to regulatory elements is unknown but appears to be independent of the SNF1 pathway.

The regulation of YGP1 appears to be unique compared with that of other genes which are subject to glucose repression. For example, the gp37 protein is not synthesized when logarithmically growing cells are shifted to 0.05 to 0.1% glucose or when cells are grown with nonfermentable carbon and energy sources. In addition, YGP1 appears to be regulated by a more general nutrient-sensing process. The synthesis of gp37 may be controlled so that gp37 is not present at high levels during vegetative growth. This idea suggests a requirement for coordination with other aspects of the cell cycle.

Increased expression of YGP1 in response to the limitation of various nutrients fits with the observation that entry into stationary phase and the accompanying physiological changes occur in response to limiting nitrogen, phosphorus, or glucose (12, 32, 41). How each of the nutrient levels regulating the expression of YGP1 is sensed and transmitted to the cell to coordinate entry into a division cycle or into stationary phase and which specific messengers and target regions are used are not known. The observation that different types of nutrient limitation lead to various levels of gp37 synthesis may suggest that multiple regulatory elements are involved.

The expression of YGP1 is regulated in response to specific nutrient levels and not by the growth rate. The latter fact was demonstrated by the lack of elevated expression when cells exited the respiro-fermentative (logarithmic) growth phase as a result of limiting histidine or limiting sulfate. This result fits with the observation that stress resistance is independent of the cell cycle (13). Alternatively, starvation for histidine or sulfate may not represent a normal physiological situation for a wild-type yeast strain. Hence, yeast cells may not respond to these types of limitations by entering stationary phase but rather may simply cease growing.

The identification and cloning of YGP1 have provided a means of examining the expression of a gene that is regulated in response to a variety of nutrient limitations. It remains to be determined whether each of these conditions of nutrient limitation is sensed in the same way and by the same regulatory elements. The control of YGP1 expression may be unique in that it is subject to a mechanism of glucose regulation that is separate from the general type of glucose repression exemplified by genes such as SUC2 (11). Similarly, given the proposed extracellular location of gp37, degradation may involve a novel type of nutrient regulation. A further analysis of YGP1 expression may contribute to our growing understanding of the cell cycle and stress resistance at the molecular level.
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